EVALUATION OF THE RESCUE POTENTIAL OF MELATONIN IN HUMAN MESENCHYMAL STEM CELL DIFFERENTIATED DOPAMINERGIC NEURONS AND ASTROCYTES

SYNOPSIS

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1. INTRODUCTION:

Parkinson's disease (PD) is the second most common neurodegenerative disease affecting approximately 1% of the population over the age of 65 years (Gibrat *et al*, 2009). It is a chronic neurodegenerative disease characterized by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta and the presence of lewy bodies (aggregates of α-synuclein) in the cytoplasm of neurons.

Although the complete mechanism of loss of these neurons remains elusive, it is known that the cause of Parkinson's disease is a combination of genetic and environmental factors. Many of the genes known to play an important role in the initiation and progression of the disease, like PARK2, PINK1 and PARK7, are genes regulating the mitochondrial health of a cell indicating that oxidative stress plays an imperative role in the pathogenesis of PD. In both familial and sporadic forms, oxidative stress is thought to be an important reason for cell death and identification of pathways involved in mitochondrial function has provided new targets for pharmacological research. Potent antioxidant molecules like melatonin could be helpful in recovery of these neurons by reduction of oxidative stress since their role in prevention of neuronal death due to oxidative stress is well known (Mayou *et al*, 2005 ;Song *et al 2015*; Mack *et al*, 2016).

Oxidative stress in neurons results from increased toxicity which is regulated by the microenvironment in which they are present. This microenvironment is maintained by the astroglial cells surrounding the neurons. Astrocytes are also known to be hampered in PD. Astrocytes, the most populous glia cells perform a range of functions which are essential for maintaining neuronal health. They produce various neurotrophic molecules like glial-derived neurotrophic factor which are especially important for the development and survival of dopaminergic neurons (Lin *et al*, 1993). Evidence is emerging to suggest that impaired astrocyte function is involved in dopaminergic neuron degeneration in PD (Booth *et al*, 2017). Also, genes regulating the mitochondrial dynamics of cell are expressed in astrocytes at levels comparable to, or in some cases higher than in, neurons (Zhang *et al*, 2016) making them important players in regulating oxidative stress.

Therefore, in order to study oxidative stress in PD, both neurons and astrocytes need to be studied.

This study has been designed to study the role of melatonin on human astrocytes and neurons (derived from human MSCs) upon exposure to 6-OHDA. This will include the identification of the genes regulated by melatonin in neurons and astrocytes.

2. REVIEW OF LITERATURE:

2.1PARKINSON'S DISEASE:

In 1817, a British physician named James Parkinson published a monograph in which he, for the first time, described a handful of patients who had PD like symptoms including tremor at rest, bradykinesia and stooped posture. Later, in 1899 Brissaud suggested that the Subtantia Nigra may be the site of lesion in PD (Przedborski, 2017).

Although the presence of Lewy bodies in PD was first described by Fritz Heinrich Lewy in 1913(Przedborski, 2017) however, it was only in 1998 that it was discovered that α-synuclein is the major component of Lewy Bodies (Krüger *et al*, 1998). As the pathophysiology of the disease became increasingly well understood, it became apparent that the cause of the disease is a combination of genetic and environmental factors. Although the exact mechanisms of PD still remain misunderstood to a certain extent, it has become clear that several biochemical abnormalities including mitochondrial dysfunction, free radical-mediated damage, inflammatory change and proteasomal abnormalities are involved (Schapira, 2007)

2.2PARKINSON'S DISEASE AND MITOCHONDRIAL DYSFUNCTION:

In 1990 it was established that mitochondrial complex I and NADH- cytochrome c reductase activity was significantly reduced in PD patients. It was also observed that this biochemical defect is the same as that produced in animal models of parkinsonism by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Przedborski, 2017). Various studies since then confirmed the presence of mitochondrial dysfunction in Parkinson's disease (Langston *et al*, 1999; Dauer *et al*, 2003). Eventually, the identification of the genes involved in PD led to a better understanding of the mechanism of oxidative stress and mitochondrial dysfunction.

The SNCA gene coding for α-synuclein was the first gene to be identified with a role in Parkinson's disease. In 1997, it was discovered that mutation in the SNCA gene is responsible for the development of Parkinson's

disease (Przedborski, 2017). In 2004, Song *et al* showed that overexpression of wild type α-synuclein results in increased mitochondrial pathology in dopaminergic neurons when exposed to low doses of MPTP. Subsequently, other genes regulating mitochondrial health which were affected in PD were also identified

The role of PARK7, coding for protein DJ-1 in PD pathophysiology was established in 2003 by Bonifati *et al*, who showed that mutation in DJ-1 was associated with early-onset of PD.

PARK2, coding for PINK1 was the next gene identified with a role in impaired mitochondrial health in PD. Palacino *et al*, in 2004 showed that PARK2 knockout mice have reduced mitochondrial complex I and complex IV activity. Further, in 2006, Rosen *et al* showed that PARK2 knockout mice showed increased sensitivity to rotenone, a pesticide known to cause PD. Yu *et al*, in 2011 showed that the PINK1/Parkin pathway regulates mitochondrial autophagy in a cell, which is known to be disrupted in PD.

Finally, the role PARK6, coding for protein PINK1 in PD was established in 2008 when Wood-Kaczmar *et al* showed that PARK6 knockout mice and human dopaminergic neurons have high ROS (Reactive oxygen species) generation and a high sensitivity to apoptosis. In 2011, Wang *et al* showed that overexpression of PARK6 in neurons restores normal mitochondrial morphology and inhibits ROS production.

2.3 MELATONIN AS A NEUROPROTECTIVE AGENT:

Melatonin, also known as *N*-acetyl-5-methoxy tryptamine, is a hormone produced by the pineal gland in animals that regulates sleep and wakefulness. Studies have also shown that melatonin functions as an antioxidant by mechanisms like direct scavenging of radicals and radical products, reduction of the activation of pro-oxidant enzymes and maintenance of mitochondrial homeostasis (Zhang *et al*, 2014)

As our understanding of neurodegeneration progressed and the role of oxidative stress became apparent, people started studying the role of antioxidants in prevention of neuronal death. The role of melatonin as a neuroprotective agent was recognized in the late 2000s. In 2007, Saravanan *et al* showed that Melatonin treatment in sparague dawley rats restores the rotenone-induced decrease in GSH level and offers neuroprotection. In 2009, Borah *et al* showed that melatonin treatment significantly reduces MPTP induced increase in levels of 6–OHDA and prevented the loss of dopaminergic neurons. However, these studies do

not comment on melatonin's ability to recover already damaged neurons. The first report of melatonin's ability to recover damaged neurons was a 2016 study by Diaz-Casado *et al* who show that melatonin can rescue zebrafish embryos from a parkinsonian phenotype produced by exposure to MPTP. This is also the first study that provides insight into the molecular targets of melatonin. There have been no other studies that show the ability of this antioxidant in rescuing neurons in a PD model.

There have also been studies on the effect of melatonin on α-synuclein. It is known that pre-treatment with melatonin prevent accumulation of α-synuclein upon administration of Amphetamine (Sae-Ung et al, 2012)

2.4THE ROLE OF ASTROCYTES IN PARKINSON'S DIESEASE

Astrocytes are critical supporters of neuronal function. They are involved in several aspects of neuronal health like glutamate clearance, maintenance of the blood brain barrier, release of several neurotrophic factors and prevention of oxidative stress (Cabezas *et al*, 2014). The neuroprotective role of astrocytes depends largely on the molecules that they release into and take up from the extracellular space, which the astrocytes and neurons share. (Rappold and Tieu, 2010). Therefore, degradation of astrocytes can lead to a toxic microenvironment which causes neuron death.

As mentioned above, many of the genes regulating the mitochondrial dynamics of cell are expressed in astrocytes at levels comparable to, or in some cases higher than in, neurons (Zhang *et al*, 2016) making them important players in regulating oxidative stress.

Booth *et al*, in a 2017 review, have commented on the various mechanisms regulated by these genes in astrocytes and how they can get affected in neurodegeneration:

1. α-synuclein from the neurons is released into the environment and taken up by astrocytes where it is broken down. However, under pathological conditions, α-synuclein expression in astrocytes increases which lead to mislocalisation of aquaporin-4 channels away from their end feet, impairing water transport. This also leads to reduced glutamate uptake resulting in glutamate

accumulation in the extracellular space causing glutamate toxicity in neurons. Glutamate uptake is also affected by the expression of DJ-1.

2. One consequence of neurodegeneration is reduced astrocyte proliferation which decreases their neuroprotective capacity. Astrocyte proliferation is regulated by the PINK1 gene which is also an important regulator of the mitochondrial dynamics.
3. Astrocytes are important for alleviating oxidative stress. This is achieved by release of molecules like Glutathione (GSH) which protect neurons. GSH release depends in part on Parkin expression in astrocytes. Increased expression of Parkin can result in improved ability of astrocytes in neuroprotection.

Therefore, it is important to take astrocytes into consideration when studying neurodegeneration.

There have been very few studies on the effect of melatonin in astrocytes. Martin *et al*, in 2002 studied the effect of melatonin on oxidative stress by GSH activity measurements and showed that melatonin prevents oxidative stress in rat astrocytes. This was confirmed by Jou *et al* in 2004 who showed that melatonin significantly reduced the mitochondrial swelling upon exposure to H_2O_2.

In a 2013 study by Mullet *et al*, it has been shown that over expression of DJ-1 in astrocytes augments their capacity for neuroprotection.

There have been no studies that probe into studying the molecules interacting with melatonin in astrocytes and its downstream effects when it is administered to prevent neurodegeneration.

2.5 DIFFERENTIATION OF MESENCHYMAL STEM CELLS INTO NEURONS AND ASTROCYTES

Mesenchymal Stromal cells or Mesenchymal Stem Cells are immunologically naïve, plastic adherent cells. They are characterized by their ability to differentiate into cells of mesodermal lineage viz. osteoblasts, chondroblasts and adipocytes; the presence of CD90, CD73 and CD 29 cell surface markers and the absence of HLA class II, C34 and CD45 surface markers (Dominici *et al*, 2006).

Neuronal differentiation potential of MSCs has been studied by several research groups using different approaches. In 2003 Jiang *et al* differentiated murine MSCs into neurons using basic fibroblast growth factor (b-FGF), Brain Derived Neurotrophic factor (BDNF) FGF8 and sonic Hedgehog (SHH). Several other studies have also shown differentiation of MSCs into neurons using a combination of different inducers like DMSO, cAMP, Epidermal Growth Factor (EGF) and Nerve Growth Factor (NGF) (Tio *et al*, 2010; Singh *et al*, 2017).

MSCs have also been shown to differentiate into astrocytes Suzuki *et al*, in 2004 that rat MSCs can be differentiated into astrocytes in the presence of BDNF and bFGF in neurobasal medium. Bahat-Stroomza *et al* in 2009 differentiated MSCs into astrocytes using a combination of IBMX, PDGF, neuregulin and EGF. Other inducers like Ciliary Neurotrophic Factor (CNTF), Bone Morphogenetic Protein-2 (BMP-2) and BMP-4 have been use to induce stem cells into astrocytes (Shaltouki *et al*, 2013).

3. OBJECTIVES

The aim of the study is to investigate the potential and underlying mechanism of melatonin, a well-known antioxidant, in recovering damaged neurons and astrocytes. In order to accomplish this aim, the following objectives have been designed:

1. To differentiate human Mesenchymal Stem Cells (MSCs) into Dopaminergic (DA) Neurons and Astrocytes.

   a. Expansion and characterization of Mesenchymal Stem Cells.

   b. Differentiation of hMSCs cells into dopaminergic (DA) neurons.

   c. Differentiation of hMSCs into astrocytes.

2. To study the effect of melatonin on human MSC differentiated astrocytes.

   a. To induce damage to the MSC differentiated neurons with 6-OHDA.

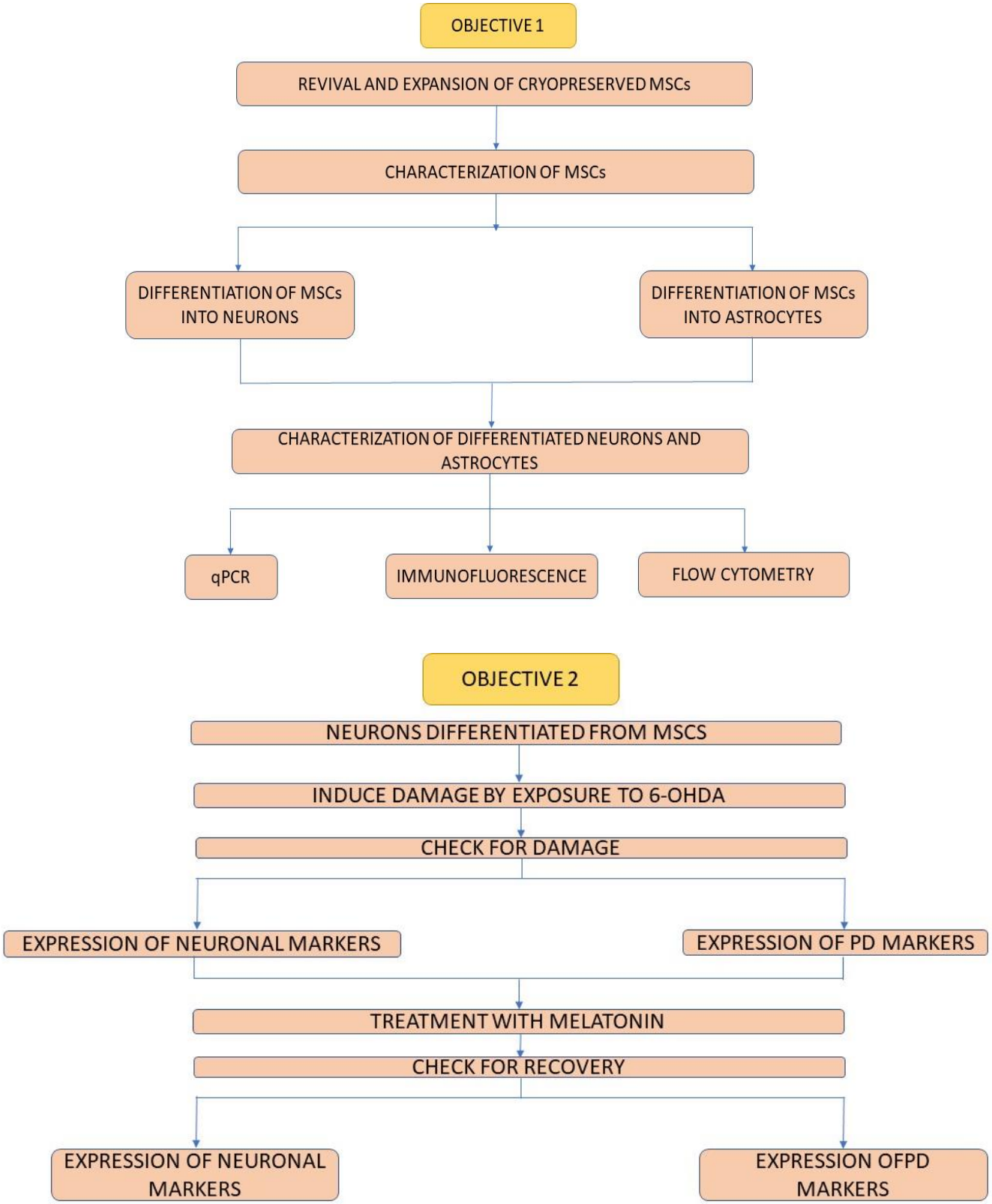
   b. To study the potential of melatonin to recover damaged neurons.

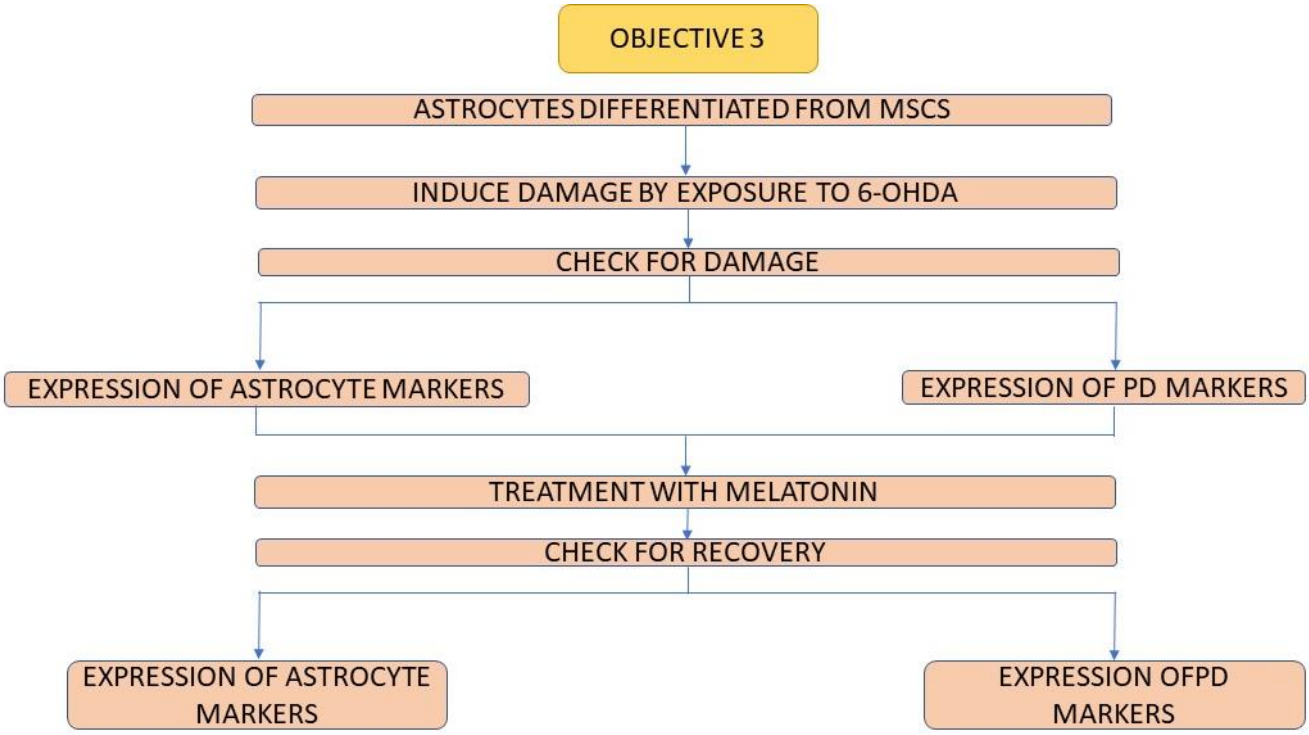
3. To study the effect of melatonin on human MSC differentiated astrocytes.

   a. To induce damage to the MSC differentiated astrocytes with 6-OHDA.

   b. To study the potential of melatonin to recover damaged astrocytes.

4. PLAN OF WORK




OBJECTIVE 3
ASTROCYTES DIFFERENTIATED FROM MSCS
INDUCE DAMAGE BY EXPOSURE TO 6-OHDA
CHECK FOR DAMAGE
EXPRESSION OF ASTROCYTE MARKERS
EXPRESSION OF PD MARKERS
TREATMENT WITH MELATONIN
CHECK FOR RECOVERY
EXPRESSION OF ASTROCYTE MARKERS
EXPRESSION OFPD MARKERS

5. MATERIALS AND METHODS:

5.1 Revival and Expansion of hMSCs:

Cryopreserved mesenchymal stem cells from dental pulp tissue (Cells will be procured from the Stem Cell Facility, All India Institute of Medical Sciences)will be revived and seeded onto the culture dish along with DMEM-LG media with 10% FBS, L- glutamine, 1X PenStrep. Cells will be passaged when the cells reach 80- 90% confluence

5.2 DIFFERENTIATION OF MSCs INTO DA NEURONS:

For neuronal differentiation, induction medium containing Neurobasal media , B27 supplement, EGF, FGF2 and BDNF and 1%PenStrep will be used (Singh *et al*, 2017). The induction protocol will be carried out for 12 days with media change on every 3rd day. After termination of induction period at 12 days, the cells will used for further experiments.

5.3 DIFFERENTIATION OF MSCs INTO ASTROCYTES:

For neuronal differentiation, induction medium containing DMEM-HG media, CNTF, BMP-4 and 1%PenStrep will be used (Magistri *et al*, 2016). The induction protocol will be carried out for 14 days with media change on every 3rd day. After termination of induction period at 14 days, the cells will used for further experiments.

5.4 TREATMENT OF 6-OHDA:

The differentiated DA neurons and astrocytes will be exposed to 6-OHDA after standardization of dose and time of exposure.

5.5 TREATMENT WITH MELATONIN:

The differentiated DA neurons and astrocytes will be exposed to melatonin after standardization of dose and time of exposure.

5.6 CHARACTERIZATION STUDIES:

Characterization studies will be done at multiple points in the study. The markers used will be as follows:

- Markers like CD73, CD90, HLA Class I, HLA Class II, CD29 and CD105 for characterization of MSCs
- Markers like MAP-2, DAT, TH for characterization of neurons
- Markers like GFAP, S100b for characterization of astrocytes
- Marker for astrocyte proliferation – Ki67
- Markers like MAP-2, TH, SNCA, PARK7, PINK1, PARK2 for damage by 6-OHDA and recovery by melatonin in neurons
- Markers like GFAP, S100b, PARK7, PINK1, PARK2 for damage by 6-OHDA and recovery by melatonin in astrocytes

The techniques that will be used to study the markers have been described below:

5.6.1 MORPHOLOGICAL ANALYSIS:

The cells will be routinely observed under inverted phase contrast microscope and images will be captured.

5.6.3 REAL-TIME QUANTITATIVE PCR (qPCR):

The qPCR experiments were performed using a Realplex real time PCR detection system (Eppendorf, Germany). Reactions will be carried out using SYBR Green Super Mix (Kapa Biosystems, USA) in a final volume of 10 µl with 0.3 µM of each primer. (Nandy *et al*, 2014)

5.6.3 MARKER PROFILING BY FLOW CYTOMETRY:

The method Single cell suspension will be prepared in media after detaching the cells from the culture flask using trypsin-EDTA solution. Cultured cells will be washed twice with 1X PBS and then will be carefully scraped using a plastic cell scraper. The cells at a concentration of $0.5–1\times10^6$/ml will be stained with the fluorochrome conjugated antibodies and will be incubated at room temperature for 1 hour in dark. The cells

will finally be acquired on an LSR II flow cytometer (Becton Dickinson) with at least 5000 events for each sample and will be analyzed with FACs DIVA (version 6.1.2).

5.6.4 IMMUNOFLUORESCENCE:

The cells will be allowed to adhere to coverslips. They will be fixed for 10 mins with chilled methanol. After washing three times with PBS, the cells will be incubated at 4ºC overnight with the primary antibodies directed against respective markers. The incubation with secondary-antibody will be at 37ºC for 1 hour 30 mins. After this, cells will be washed with PBS 12-15 times and then nuclei will be counter-stained with DAPI for 5 minutes. Finally, the cells will be mounted for microscopic examination.

- Part of the experimental work will be done at the Stem Cell Facility (DBT-centre of excellence), All India Institute of Medical Sciences, New Delhi.

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